

## Immunosuppressive Effects of *Prevotella intermedia* on In Vitro Human Lymphocyte Activation

BRUCE J. SHENKER,<sup>1,2\*</sup> LAURA VITALE,<sup>1,2</sup> AND JORGEN SLOTS<sup>2,3</sup>

Departments of Pathology<sup>1</sup> and Periodontics<sup>3</sup> and the Periodontal Diseases Research Center,<sup>2</sup> University of Pennsylvania School of Dental Medicine, 4010 Locust Street, Philadelphia, Pennsylvania 19104-6002

Received 16 May 1991/Accepted 26 September 1991

In this study, we have assessed four strains of *Prevotella intermedia*, isolated from periodontally involved lesions, for their ability to inhibit lymphocyte functions. All four strains were found to cause a dose-dependent inhibition of B- and T-cell proliferation in response to mitogens and antigens. This was reflected in altered DNA, RNA, and protein syntheses. Furthermore, *P. intermedia* appeared to affect the early stages of cell activation. This was ascertained by kinetic analysis in which it was determined that the extract had to be present during the first 24 h of incubation to cause suppression. Moreover, direct assessment of the early stages of cell activation indicated that release of cytokines and expression of the interleukin 2 receptor and CD69 on T cells were inhibited by *P. intermedia* sonic extracts. Finally, preliminary characterization of the immunosuppressive agent indicates that it has a molecular mass of approximately 50 kDa and is heat labile. It has been proposed that impaired host defense may play a pivotal role in the pathogenesis of many infections. The data presented in this paper suggest that microbially mediated immunosuppression may contribute to the pathogenesis of periodontal disease by altering the nature and consequences of host-parasite interactions.

Microbial virulence may be the consequence of several properties, including the ability of certain species to resist, escape, or pervert host defense mechanisms. Such mechanisms have been implicated in the pathogenesis of several diseases, including measles (11), influenza (1), leprosy (7), candidiasis (9), leishmaniasis (14), trypanosomiasis (2), cryptococcosis (4), tuberculosis (12), and syphilis (27), among others. Perhaps the most prominent example of this relationship between host and pathogen is AIDS, where the causative virus infects and destroys a subpopulation of T lymphocytes. While the nature and contribution of the immune system to the pathogenesis of periodontal disease are poorly understood, recent studies suggest that the development of these disorders may be associated with immunologic dysfunction (reviewed in reference 16). Although the basis for this dysfunction is unclear, we have found that several putative periodontal pathogens are capable of inhibiting human lymphocyte responsiveness (17-21). The ability of microorganisms to suppress the immune response of the host may affect the course of initial infection by facilitating spread, multiplication, and persistence and may lead to enhanced susceptibility to infection by secondary pathogens (reviewed in references 15 and 28). Previously, we demonstrated that a nonoral isolate of *Prevotella intermedia* was capable of suppressing lymphocyte proliferation (22). We have now extended these studies to include oral isolates of *P. intermedia* and to further characterize the immunosuppressive properties of this organism.

### MATERIALS AND METHODS

**Bacterial strains and culture conditions.** The *P. intermedia* strains used in this study, M87-903, M87-679, M87-714, and M87-735, were fresh isolates from advanced periodontitis lesions of adult patients. The organisms were identified to species level by the methods of Holdeman et al. (5). All strains were grown in 1-liter cultures in 85% N<sub>2</sub>-10% H<sub>2</sub>-5%

CO<sub>2</sub> for 2 to 3 days at 37°C. The medium used was 3.7% brain heart infusion broth (Difco Laboratories, Detroit, Mich.) supplemented with 0.0005% hemin, 0.0005% menadione, 0.5% yeast extract, and 0.05% L-cysteine hydrochloride.

Bacterial fractions were prepared as previously described (22). Briefly, bacterial cells harvested from 1-liter cultures were washed, suspended in 20 ml of phosphate-buffered saline (PBS), and disrupted by sonication at 4°C for a total of 10 min with 1-min pulses in the presence of glass beads. After settling of the beads, unbroken cells and the membrane fraction were sedimented at 85,000 × g for 60 min. The protein that remained in suspension after high-speed centrifugation was designated the sonic extract (SE) and contained both cytoplasmic and periplasmic proteins. The SE was extensively dialyzed against PBS and then against RPMI 1640, and the protein concentration was determined by the Bio-Rad assay (Bio-Rad Laboratories, Richmond, Calif.) with bovine serum albumin as a standard.

**Preparation of HPMC.** Human peripheral blood mononuclear cells (HPMC) were prepared as described previously (23). Briefly, HPMC were prepared from 100 to 200 ml of heparinized venous blood of healthy donors. The blood was first centrifuged at 300 × g for 15 min at 5°C; the plasma was then removed, and the cells were brought back to the original volume with Hanks balanced salt solution (HBSS). HPMC were then isolated by buoyant density centrifugation on Ficoll-Hypaque (Pharmacia LKB Biotechnology, Piscataway, N.J.). The HPMC were washed twice with HBSS and diluted to 2 × 10<sup>6</sup> viable cells per ml of HBSS. Viable cell counts were performed by assessing trypan blue exclusion.

Purified populations of lymphocytes and monocytes were obtained by counterflow centrifugal elutriation as previously described (23). HPMC (2 × 10<sup>8</sup> to 4 × 10<sup>8</sup>) were suspended in 20 ml of HBSS-CaMg containing 0.5% bovine serum albumin (BSA) and pumped into a Beckman elutriator rotor (model JE-6 rotor equipped with a standard chamber; Beckman Instruments, Fullerton, Calif.); the flow rate was 7 ml/min, and the rotor speed was 1,960 rpm. This initial flow

\* Corresponding author.

rate permitted the mononuclear cells in the horizontal chamber to stratify by size and density while contaminating platelets were removed. Cells were then eluted by sequentially increasing the flow rate; lymphocytes were obtained at a flow rate of 7 to 12 ml/min, while monocytes were eluted at 14 to 16 ml/min. All fractions were counted, and their size distribution was monitored on a model ZBI Coulter Counter connected to an IBM personal computer with appropriate hardware and software (Personal Computer Analyzer; Nucleus Inc., Oak Ridge, Tenn.). Cells were stained with anti-CD3 (Ortho Diagnostic Systems, Raritan, N.J.), anti-human immunoglobulin (Ig) (Southern Biotechnology Associates, Birmingham, Ala.), and anti-LeuM3 (Becton Dickinson Immunocytometry Systems, San Jose, Calif.) antibodies conjugated with fluorescein isothiocyanate, and purity was assessed by immunofluorescence and fluorescence-activated cell sorter analysis. The lymphocyte and monocyte preparations were routinely found to be >98% and 85 to 95% pure, respectively. T cells were further purified by E-rosette formation. Briefly, sheep erythrocytes were washed and treated with 0.14 M 2-aminoethylisothiuronium bromide at pH 9 for 15 min. After the cells were washed four times, sheep erythrocytes and lymphocytes (isolated by counterflow centrifugal elutriation) were pelleted and incubated together for 60 min. The cells were then gently resuspended; nonrosetted cells were separated from rosetted cells by centrifugation on Ficoll-Hypaque (see above). The rosetted cells in the pellet were lysed to remove sheep erythrocytes and found to contain >98% T cells when stained with anti-CD3 monoclonal antibody and analyzed by flow cytometry.

**Assays of mitogen-induced DNA, RNA, and protein syntheses.** An HPMC suspension (0.1 ml) containing  $2 \times 10^5$  cells was placed into each well of flat-bottom microculture plates. Each culture received 0.1 ml of medium (as described above) or various concentrations of SE diluted in medium and sterilized by passage through a 0.22- $\mu$ m-pore-size Millipore filter. After 30 min of incubation at 37°C, the cells received an optimal mitogenic dose of either concanavalin A (ConA) (5  $\mu$ g/ml; Calbiochem), phytohemagglutinin (PHA) (1  $\mu$ g/ml; Wellcome Research Labs, Beckenham, England), pokeweed mitogen (PWM) (10  $\mu$ g/ml; Calbiochem), or formalinized *Staphylococcus aureus* (10  $\mu$ g of cells per ml; Pansorbin, Calbiochem). The cells were incubated at 37°C in humidified air containing 5% CO<sub>2</sub>.

Mitogen-induced DNA, RNA, and protein syntheses were assayed by the incorporation of [<sup>3</sup>H]thymidine ([<sup>3</sup>H]TdR), [<sup>3</sup>H]uridine, and [<sup>3</sup>H]leucine, respectively. For the assessment of DNA synthesis, cell cultures were incubated for 96 h as described above, and 0.25  $\mu$ Ci of [<sup>3</sup>H]TdR (specific activity, 42 Ci/mmol; Amersham, Arlington Heights, Ill.) was added for the last 4 h. The cultures were then harvested onto glass fiber filter paper with an automatic cell harvester (model PhD; Cambridge Technology, Cambridge, Mass.). [<sup>3</sup>H]TdR incorporation was determined by counting in a Beckman LS3801 liquid scintillation spectrometer. Incorporation into cells exposed to the SE was expressed as a percentage of incorporation observed in control cultures receiving mitogen alone.

RNA and protein syntheses were assessed on cells that were incubated for 48 h as previously described (19). [<sup>3</sup>H]uridine (specific activity, 28 Ci/mmol; Amersham) and [<sup>3</sup>H]leucine (specific activity, 58 Ci/mmol; Amersham) were added (0.5  $\mu$ Ci) for the last 18 h of incubation. Cells were harvested (as described above) after treatment with cold 5%

trichloroacetic acid. Incorporation was determined and expressed as described above.

**Generation and assay for IL-2.** Cultures (1.0 ml) were established containing  $2 \times 10^6$  purified T cells,  $10^5$  monocytes, various amounts of SE, and 1  $\mu$ g of PHA; cells were prepared as described above. Culture supernatants were harvested after 48 h of incubation by centrifugation. The amount of interleukin 2 (IL-2) present in the supernatant was assayed by testing multiple dilutions of each supernatant for its ability to sustain the growth of an IL-2-dependent cell line (CTLL-20), kindly provided by Phil Simon (Smith Kline and Beckman Laboratories). IL-2 activity in each sample was expressed as units by comparison to a recombinant IL-2 standard (Boehringer Mannheim Biochemicals, Indianapolis, Ind.) containing 10,000 U/ml. One unit is defined as the reciprocal of the IL-2 dilution which produces 50% maximal incorporation of [<sup>3</sup>H]TdR into IL-2-dependent CTLL cells. Since IL-2-dependent cell lines have been shown to respond to IL-4 as well as IL-2, our data are presented as units of IL-2 or IL-4 activity. Nevertheless, we have verified our results by employing a commercially available enzyme-linked immunosorbent assay (ELISA) for IL-2 (R and D Systems, Minneapolis, Minn.).

**Assessment of IL-2 receptor (IL-2R) and CD69 expression.** T cells ( $5 \times 10^6$  cells per ml) and monocytes ( $5 \times 10^5$ ) were incubated for 24 h in the presence or absence of PHA and SE. After incubation, the T cells were washed three times and divided into two aliquots. One aliquot was incubated with anti-IL-2R monoclonal antibody (anti-CD25; Becton Dickinson Immunocytometry Systems) or anti-CD69 antibody (Leu23; Becton Dickinson Immunocytometry Systems) conjugated with fluorescein isothiocyanate, and the other aliquot was incubated with a control monoclonal antibody; anti-CD69, anti-CD25, and control antibodies were all of the IgG<sub>1</sub> isotype. After 30 min on ice, the cells were washed three times and fixed overnight in 1% paraformaldehyde. Cells were examined for fluorescence staining with a fluorescence-activated cell sorter (FACStar<sup>Plus</sup> flow cytometer; Becton Dickinson Immunocytometry Systems). The flow cytometer was equipped with logarithmic amplifiers for fluorescence measurements. Fluorescein isothiocyanate was excited by an argon laser at 488 nm (200 mW), and its fluorescence was measured through a 515- to 540-nm bandpass filter. Ten thousand cells were examined for each sample; cells which gave fluorescence signals brighter than those seen on 95 to 99% control cells were considered to be positive. Data were analyzed on a Hewlett-Packard computer (Hewlett-Packard, Sunnyvale, Calif.).

**Ion-exchange chromatography.** After dialysis against 10 mM Tris buffer (pH 7.0) containing 10 mM NaCl and 1 mM EDTA, the SE was applied to a Mono Q column (0.5 by 5 cm; Pharmacia LKB Biotechnology) pre-equilibrated in this buffer. The column was then extensively washed and eluted with a linear NaCl gradient (10 mM to 0.5 M) at a flow rate of 1 ml/min. Fractions (1 ml) were collected and monitored for both A<sub>280</sub> and for immunosuppressive potential at multiple dilutions. This allowed calculation of 50% infective dose (ID<sub>50</sub>) units per milliliter by determining the amount of sample (in microliters) required to reduce [<sup>3</sup>H]TdR incorporation to 50% of control values. Appropriate fractions were pooled and concentrated by membrane ultrafiltration (YM-10 and Centricon 10; Amicon, Lexington, Mass.).

**Gel filtration chromatography.** Pooled and concentrated fractions from ion-exchange chromatography were further fractionated by gel filtration chromatography on a Superose 12 column (Pharmacia). The column was equilibrated with

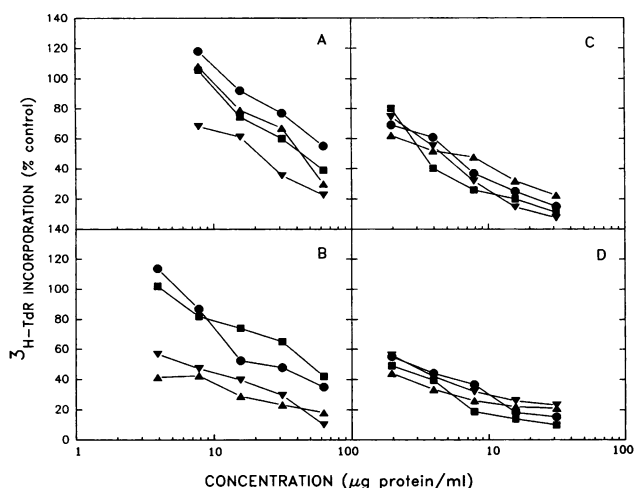


FIG. 1. Effects of *P. intermedia* on the proliferative response of HPMC to mitogens. HPMC were incubated with SE prepared from each of four *P. intermedia* isolates: M87-903 (circles), M87-735 (squares), M87-714 (inverted triangles), and M87-679 (triangles). At 30 min of incubation, an optimal mitogenic dose of PHA (A), ConA (B), PWM (C), or *S. aureus* (D) was added, and, after 4 days, proliferation was assessed by [ $^3$ H]TdR incorporation. The results are plotted as a percentage of [ $^3$ H]TdR incorporation observed in control cultures receiving mitogen alone. Each point represents the mean of at least three experiments, each performed in quadruplicate culture. Net [ $^3$ H]TdR incorporation in cells receiving mitogen averaged 62,745 cpm (PHA), 32,423 cpm (ConA), 46,415 cpm (PWM), and 1,398 cpm (*S. aureus*); incorporation into control cultures (no mitogen) averaged 226 cpm. Standard errors were within 5% of the mean.

PBS; a 200- $\mu$ l sample was applied, and 0.5-ml fractions were collected. All fractions were monitored for  $A_{280}$  and for their ability to suppress lymphocyte proliferation.

## RESULTS

Bacterial extracts were first evaluated for their ability to alter HPMC responses to several mitogens, including PHA, ConA, PWM, and *S. aureus*. As shown in Fig. 1, all four strains were capable of inhibiting mitogen-induced [ $^3$ H]TdR incorporation. Responses to all four mitogens were inhibited in a dose-dependent fashion; PWM- and *S. aureus*-induced activations were consistently found to be more sensitive to the inhibitory effects of the *P. intermedia* SEs than activation induced by either PHA or ConA. As shown in Table 1, ID<sub>50</sub> values (dose of SE [in micrograms of protein per milliliter] required to reduce [ $^3$ H]TdR incorporation to 50%

TABLE 1. Comparison of the immunosuppressive potential of four *P. intermedia* isolates

Mitogen	ID <sub>50</sub> value <sup>a</sup> for isolate:			
	M87-903	M87-735	M87-714	M87-679
PHA	87.0	47.8	25.5	63.5
ConA	36.6	50.5	5.4	6.1
PWM	7.1	4.4	5.5	6.7
<i>S. aureus</i>	4.5	1.8	1.7	0.8

<sup>a</sup> ID<sub>50</sub> values are the amounts of SE (micrograms of protein per milliliter) required to reduce mitogen-induced [ $^3$ H]TdR incorporation to 50% of control values.

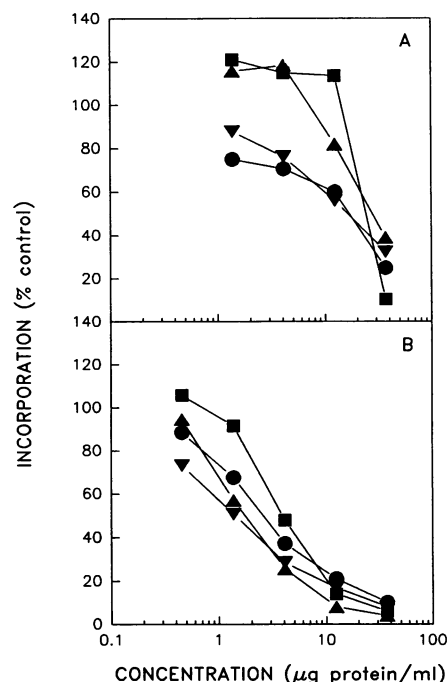


FIG. 2. Effect of *P. intermedia* on RNA and protein syntheses induced by ConA. HPMC were exposed to SE as described in the text, and protein synthesis (A) and RNA synthesis (B) were assessed by [ $^3$ H]leucine and [ $^3$ H]uridine incorporation, respectively. Four isolates of *P. intermedia* were employed: M87-903 (circles), M87-735 (squares), M87-714 (inverted triangles), and M87-679 (triangles). The results are plotted as a percentage of radiolabelled precursor incorporation in control cultures receiving ConA only. Each point represents the mean value of quadruplicate cultures of each of three experiments; standard errors were within 5% of the mean. Net incorporation in cells receiving ConA alone averaged 751 cpm and 1,695 cpm for [ $^3$ H]leucine and [ $^3$ H]uridine, respectively. Incorporation into control cultures (no mitogen) averaged 138 cpm ([ $^3$ H]leucine) and 1,056 cpm ([ $^3$ H]uridine).

of control values) were lower for PWM and *S. aureus* than those observed when either PHA or ConA was employed as the mitogenic stimulant. This difference in sensitivity may, in part, reflect differences in cell susceptibility to the SE since PWM and *S. aureus* predominantly activate B cells and PHA and ConA activate T cells. These inhibitory effects were not simply due to cell death; the SE had no effect on cell viability, as evidenced by trypan blue exclusion at the concentrations employed. In addition to mitogen responsiveness, antigenic responses to tetanus toxoid and streptokinase/streptodornase were similarly inhibited (data not shown).

Experiments were also performed to determine if the four SEs could suppress other parameters of lymphocyte activation. Figure 2 shows the results of experiments in which RNA and protein syntheses were assessed in HPMC after exposure to SE and ConA. These results clearly indicate that both metabolic processes were inhibited, with RNA synthesis being more sensitive than protein synthesis. Not only was there a generalized decrease in protein synthesis ([ $^3$ H]leucine incorporation), but the ability of B cells to synthesize and secrete IgG and IgM in response to PWM was also reduced. As shown in Table 2, strain M87-903 and M87-714 SEs caused a dose-dependent reduction in the ability of

TABLE 2. Effect of *P. intermedia* on PWM-induced Ig production

Culture conditions	Ig production <sup>a</sup> of isolate:			
	M87-903		M87-714	
	IgG	IgM	IgG	IgM
Cells only	18	0	18	0
PWM only	2,080 (0)	170 (0)	2,080 (0)	170 (0)
PWM + 6.25 µg of SE	1,087 (48)	109 (36)	1,680 (19)	190 (112)
PWM + 12.50 µg of SE	624 (70)	52 (69)	556 (73)	39 (77)
PWM + 25.00 µg of SE	103 (95)	28 (84)	353 (83)	40 (76)

<sup>a</sup> Data are expressed as the amount of IgG or IgM produced (in nanograms per milliliter) in culture supernatants harvested after 8 days; numbers in parentheses represent the percent inhibition based upon Ig produced in control (PWM) cultures. Results represent the mean of triplicate cultures from a representative experiment; standard deviations were within 10% of the mean.

human B cells to synthesize and secrete both IgG and IgM. Similar results were obtained with the other strains.

Having established that SEs obtained from all four strains of *P. intermedia* were capable of suppressing B- and T-cell responses, we next determined the conditions that result in maximal suppression of lymphocyte proliferation. Figure 3 demonstrates the temporal relationship between exposure to ConA and exposure to SE from *P. intermedia* M87-903 and their effect on cell proliferation. Maximal inhibition (80%) was observed regardless of whether SE was added 60 min prior to ConA or up to 4 h after the addition of mitogen. However, virtually no reduction in [<sup>3</sup>H]TdR incorporation was observed when the SE was added at 24 h. We were also able to demonstrate that the SE was not required to be present for the entire 4-day incubation period. In these experiments (data not shown), cells remained suppressed when they were exposed to SE for 30 min, washed, and

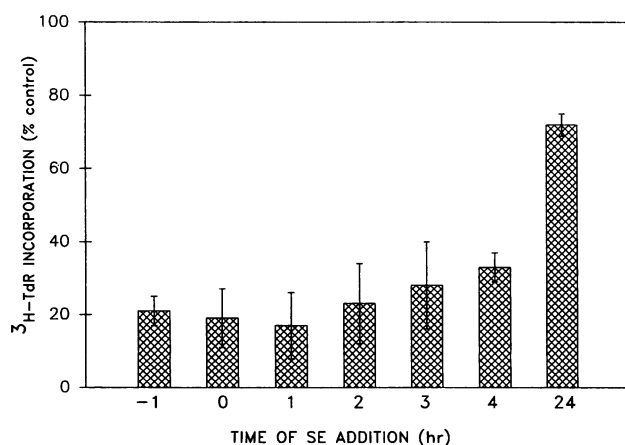


FIG. 3. The effect of pre- and post-ConA exposure to *P. intermedia* M87-903 on HPMC proliferation. HPMC were incubated with 50 µg of SE per ml for various periods of time before and after the addition of an optimal mitogenic dose of ConA (added at time zero). The cells were then incubated for 96 h after the addition of ConA. Results are plotted as a percentage of [<sup>3</sup>H]TdR incorporation in cultures receiving ConA alone. Each bar represents the mean ± standard error of quadruplicate assays of each of three experiments. Net incorporation of [<sup>3</sup>H]TdR in cells receiving ConA alone averaged 27,361 cpm; incorporation in control cultures (no mitogen) averaged 482 cpm.

TABLE 3. Effect of *P. intermedia* on cytokine production

Culture conditions	IL-2 or IL-4 production (U/ml) <sup>a</sup>	
	Expt 1	Expt 2
Medium only	0	0
PHA	31.5 (0)	26.5 (0)
PHA + 12.5 µg of SE	29.0 (8)	19.4 (7)
PHA + 25.0 µg of SE	18.8 (40)	14.2 (58)
PHA + 50.0 µg of SE	11.0 (65)	10.5 (65)

<sup>a</sup> Data are the representative results of two experiments performed with *P. intermedia* M87-903. Results are the mean of triplicate cultures; data are expressed as IL-2 or IL-4 activity because the bioassay employed cannot discriminate between the two cytokines. Numbers in parentheses represent the percent inhibition in relation to control (PHA) cultures.

returned to culture. These results suggest that *P. intermedia* affects the early stages of cell activation. This conclusion is further supported by the finding that the *P. intermedia* SEs also inhibit the production and expression of IL-2 and its receptor (IL-2R) on T cells. T-cell activation has been found to be dependent upon the synthesis and secretion of these molecules during the first 24 to 48 h after exposure to mitogen or antigen (3). As shown in Table 3, *P. intermedia* M87-903 caused a dose-dependent reduction in PHA-induced cytokine production. Similar results were observed with the other three strains of *P. intermedia*.

Figure 4 demonstrates the effect of *P. intermedia* M87-903 on PHA-induced expression of IL-2R and CD69 (Leu23). As indicated above, expression of IL-2R is an early requisite of T-cell activation. CD69 is a phosphorylated 28- to 32-kDa disulfide-linked homodimer expressed on the surface of activated T cells, B cells, and NK cells (26). This surface marker is expressed 2 to 24 h after stimulation and has been proposed as an early marker of cell activation. The SE caused a reduction in the percentage of cells expressing IL-2R from 40% (PHA control) to 22% (SE treated). In those cells still expressing IL-2R after exposure to *P. intermedia*, the surface density of the receptor was reduced, as evidenced by a decrease in the fluorescence intensity. Likewise, the SE caused a reduction in the percentage of cells expressing CD69, from 49% in PHA-treated cells to 21% in cells exposed to the bacterial extract.

Preliminary studies have been carried out to determine the physicochemical characteristics of the *P. intermedia* suppressive substance. The inhibitory activity was found to be temperature sensitive (Fig. 5), with partial degradation (or neutralization) of the inhibitor occurring after a 30-min exposure to a temperature of 56°C and total destruction after exposure to 85°C. The crude SE was also fractionated by ion-exchange chromatography (Mono Q); the activity bound to the column and could be eluted with ~0.2 M NaCl (Fig. 6). The active fractions were pooled, concentrated, and applied to a Superose 12 gel filtration column. As shown in Fig. 7, the suppressive activity appeared in one well-defined peak corresponding to a molecular mass of approximately 50 kDa.

## DISCUSSION

In previous studies, we demonstrated that SE prepared from *P. intermedia*, *Porphyromonas asaccharolytica*, *Porphyromonas endodontalis*, and *Prevotella melaninogenica* were capable of suppressing human T- and B-cell responsiveness (22). This report extends those observations to include assessment of the immunomodulatory capability of

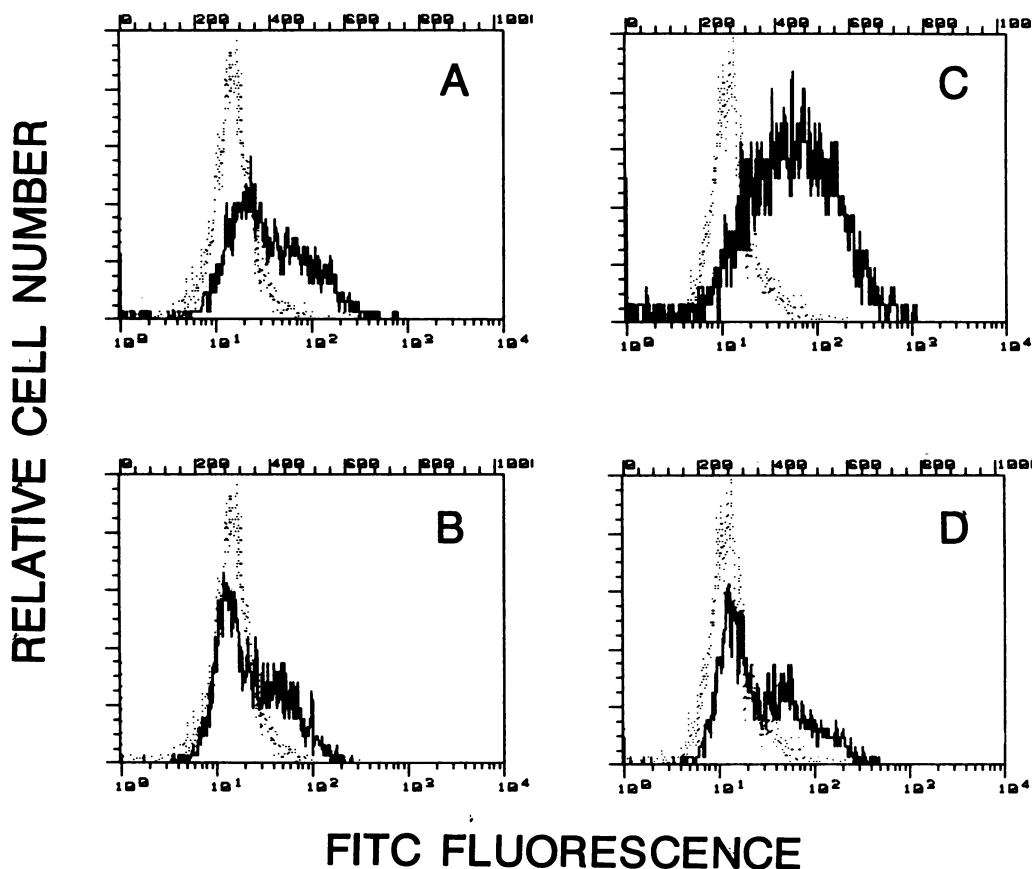


FIG. 4. Flow cytometric analysis of surface IL-2R and CD69 expression. T cells were incubated with PHA in the presence of *P. intermedia* M87-903 SE (50  $\mu$ g/ml) for 24 h. The cells were processed for fluorescence-activated cell sorter analysis as described in Materials and Methods. Exposure to an optimal PHA concentration resulted in 40% IL-2R-positive cells (A) (mean channel fluorescence, 529) and 49% CD69-positive cells (C) (mean channel fluorescence, 552). Cultures receiving both PHA and SE resulted in a reduction to 22% IL-2R-positive cells (B) (mean channel fluorescence, 481) and 21% CD69-positive cells (D) (mean channel fluorescence, 526). Dotted lines represent fluorescence of cells stained with isotypic control antibodies. These data are representative of three separate experiments.

oral isolates of *P. intermedia* and to further characterize the inhibitory effects. All four *P. intermedia* strains inhibited mitogen (and antigen)-induced activation of both human B and T cells. Inhibition was dose dependent, and B cell responsiveness (to PWM and *S. aureus*) was found to be considerably more sensitive to the bacterial extracts than were T cells (to ConA and PHA). Suppression was reflected in altered protein, RNA, and DNA syntheses. Preliminary characterization suggests that the immunosuppressive activity is nondialyzable and heat labile, with activity being completely destroyed at 85°C. Gel filtration chromatography of active material eluted from an ion-exchange column yielded a single, well-defined peak of inhibitory activity corresponding to a molecular mass of 55 kDa.

Microbial products represent an important source of immunoregulatory agents. In particular, several microorganisms are capable of suppressing the immune response through various products, including toxins, enzymes, cell wall components, and metabolites (for reviews, see references 15 and 28). These immunosuppressive products may alter the immune system via different mechanisms. In some instances these agents indirectly modify lymphocyte responsiveness by directly affecting monocyte or macrophage activities. For example, *Treponema denticola*, *Treponema pallidum*, and *Corynebacterium parvum* provoke the release

of hydrogen peroxide, prostaglandin, and other mediators capable of inhibiting lymphocyte function (10, 20, 27). Other organisms such as *Leishmania donovani* impair the synthesis and release of IL-1 from macrophages (14). Recently, we have demonstrated that a toxin produced by *Actinobacillus actinomycetemcomitans*, a periodontal pathogen, is capable of impairing lymphocyte responsiveness by selectively killing monocytes (13). In addition to affecting monocytes, several microorganisms interact directly with lymphocytes, resulting in nonspecific inhibition of their activation and/or proliferation. For example, cholera toxin interacts with lymphocytes in vitro and in vivo and activates adenylate cyclase which results in intracellular accumulation of cyclic AMP (6, 24). *Trypanosoma cruzi*, on the other hand, has been shown to impair lymphocyte activation by interfering with IL-2R expression (2). Other microbial factors target subpopulations of regulatory T cells or monocytes, such as human immunodeficiency virus, which infects and destroys helper T cells. We have also demonstrated that *A. actinomycetemcomitans* produces an immunosuppressive factor capable of activating suppressor T cells (17). Similarly, *Cryptococcus neoformans* has been shown to induce suppressor T cells which result in the subsequent impairment of delayed-type hypersensitivity responses in vivo (8).

*P. intermedia* clearly impairs both B- and T-cell activa-

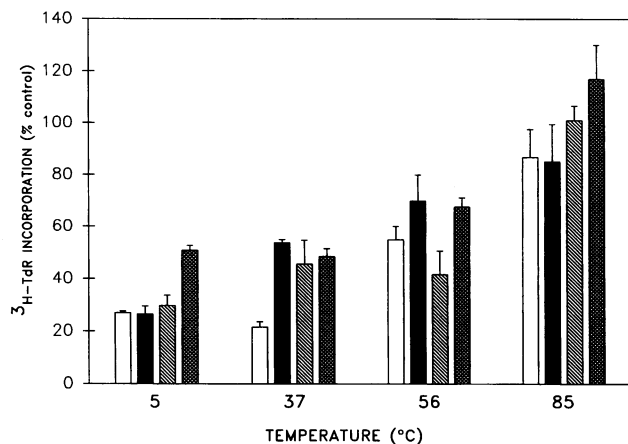


FIG. 5. Effect of temperature on *P. intermedia* inhibitory activity. Temperature sensitivity of the lymphocyte inhibitory activity was determined by exposing SEs of each isolate (M87-903 [open bars], M87-735 [solid bars], M87-714 [hatched bars], and M87-679 [cross-hatched bars]) to different temperatures for 30 min and then reequilibrating them to 37°C. The samples were then added to lymphocyte cultures at a final concentration of 25 µg/ml as described in Materials and Methods. Results are plotted as a percentage of [ $^3\text{H}$ ]TdR incorporation in cultures receiving ConA alone. Each point represents the mean  $\pm$  standard error of quadruplicate cultures of a representative experiment. Net [ $^3\text{H}$ ]TdR incorporation in cultures receiving ConA alone averaged 29,448 cpm.

tion. Our current observations do suggest direct effects on the early stages of T-cell activation as evidenced by a reduction in IL-2 (and/or IL-4) production and by the expression of IL-2R and CD69. The role and requirement for IL-2 in the activation of lymphocytes (both B and T) is well established (25). Likewise, the importance of expression of IL-2R during the early stages of the activation process has been documented (3). IL-2 plays an important role in paracrine and autocrine regulatory processes and thereby influences the expression of IL-2R (3). Therefore, the ability of *P. intermedia* to impair the production of both IL-2 and IL-2R should account for the observed suppression of T- and B-cell activation and proliferation. At this point, the actual

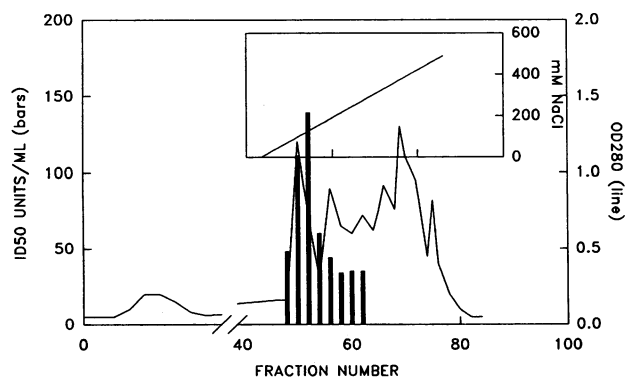


FIG. 6. Ion-exchange chromatography of *P. intermedia* SE. SEs were prepared from M87-903 as described in Materials and Methods and applied to a Mono Q column. Activity was eluted with a linear NaCl gradient. The profile of optical density at 280 nm (line) is indicated; alternate fractions were assayed for their ability to inhibit lymphocyte proliferation in response to ConA (solid bars), and the results were plotted as ID<sub>50</sub> units per milliliter.

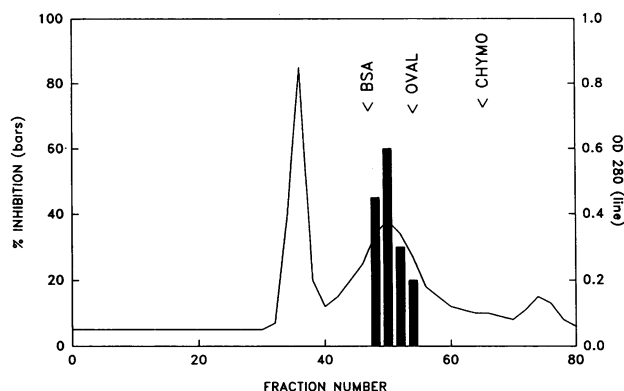


FIG. 7. Gel filtration chromatography of *P. intermedia* SE. Active fractions from the Mono Q column were pooled, concentrated, and applied to a Superose 12 column equilibrated in PBS. The profile of optical density at 280 nm (solid line) along with molecular weight markers (BSA, OVAL, and CHYMO) are indicated. Alternate fractions were assayed for their ability to inhibit lymphocyte proliferation to ConA (solid bars), and the results were plotted as percent inhibition with respect to cultures receiving mitogen alone.

mechanism by which *P. intermedia* impairs the expression of these proteins is not known. While IL-1 has been shown to be critical for the production of these regulatory proteins, preliminary studies do not indicate a role for the monocyte in mediating the suppressive effects of *P. intermedia*. Studies are currently under way to determine if the primary effect of *P. intermedia* involves IL-2 or some preceding event that ultimately results in reduced production of IL-2, IL-2R, and CD69. In this regard, expression of these proteins has been linked to early events of lymphocyte activation associated with membrane signal transduction such as  $\text{Ca}^{2+}$  influx, protein kinase C activation, or both (26).

In conclusion, the ability of microorganisms to modulate in vivo and in vitro immunological responsiveness is an established phenomenon. The exact contribution of these exogenous regulatory processes to periodontal disease remains to be determined. Our current finding that *P. intermedia* inhibits the immune response suggests that impaired host defense mechanisms may contribute to the disease process. Such inhibitory factors could lead to a state of immunological hyporesponsiveness that favors colonization by the initiating organism or by other opportunistic organisms.

#### ACKNOWLEDGMENTS

We thank Caroline Rooney and Elizabeth Ruggieri for their excellent technical assistance.

These studies were supported by National Institutes of Health grants DE06014, DE08587, DE07118, and DE00170.

#### REFERENCES

1. Beck, M. A., and J. F. Sheridan. 1989. Regulation of lymphokine response during reinfection by influenza virus. Production of a factor that inhibits lymphokine activity. *J. Immunol.* **142**:3560-3567.
2. Beltz, L. A., F. Kierszenbaum, and M. B. Szein. 1989. Selective suppressive effects of *Trypanosoma cruzi* on activated human lymphocytes. *Infect. Immun.* **57**:2301-2305.
3. Cantrell, D. A., M. K. L. Collins, and M. J. Crumpton. 1988. Autocrine regulation of T-lymphocyte proliferation: differential induction of IL-2 and IL-2 receptor. *Immunology* **65**:343-349.
4. Fidel, P. L., Jr., and J. W. Murphy. 1988. Characterization of an in vitro-stimulated, *Cryptococcus neoformans*-specific second-order suppressor T cell and its precursor. *Infect. Immun.*

- 56:1267-1272.
5. Holdeman, L. V., E. P. Cato, and W. E. C. Moore. 1977. Anaerobic laboratory manual, 4th ed. Virginia Polytechnic Institute Anaerobe Laboratory, Virginia Polytechnic Institute and State University, Blacksburg.
6. Holmgren, J., and L. Lindholm. 1977. Cholera toxin, ganglioside receptors and the immune response, p. 77-96. In B. Cinader (ed.), Immunology of receptors, immunology series. Marcel Dekker, Inc., New York.
7. Kaplan, G., R. R. Gandhi, D. E. Weinstein, W. R. Levis, M. E. Patarroyo, P. J. Brennan, and Z. A. Cohn. 1987. Mycobacterium leprae antigen-induced suppression of T cell proliferation in vitro. J. Immunol. 138:3028-3034.
8. Khakpour, F. R., and J. W. Murphy. 1987. Characterization of a third-order suppressor T cell (Ts3) induced by cryptococcal antigen(s). Infect. Immun. 55:1657-1662.
9. Lombardi, G., D. Vismara, E. Piccolella, V. Colizzi, and G. L. Asherson. 1985. A non-specific inhibitor produced by Candida albicans activated T cells impairs cell proliferation by inhibiting interleukin-1 production. Clin. Exp. Immunol. 60:303-310.
10. Metzger, Z., J. T. Hoffeld, and J. J. Oppenheim. 1980. Macrophage mediated suppression. I. Evidence for participation of both hydrogen peroxide and prostaglandins in suppression of murine lymphocyte proliferation. J. Immunol. 124:983-988.
11. Olson, G. B., P. B. Dent, W. E. Rawls, M. A. South, J. R. Montgomery, J. L. Melnick, and R. A. Good. 1968. Abnormalities of in vitro lymphocyte responses during rubella virus infections. J. Exp. Med. 128:47-68.
12. Parra, C., L. F. Montaña, M. Huesca, I. Rayón, K. Willms, and F. Goodsaid. 1986. Inhibition of mitogenesis induced by phytohemagglutinin and Lens culinaris lectin in adherent-cell supernatants treated with protein extract of Mycobacterium tuberculosis. Infect. Immun. 52:309-313.
13. Rabie, G., E. T. Lally, and B. J. Shenker. 1988. Immunosuppressive properties of Actinobacillus actinomycetemcomitans leukotoxin. Infect. Immun. 56:122-127.
14. Reiner, N. E. 1987. Parasite accessory cell interactions in murine leishmaniasis. I. Evasion and stimulus-dependent suppression of the macrophage interleukin 1 response by Leishmania donovani. J. Immunol. 138:1919-1925.
15. Schwab, J. H. 1975. Suppression of the immune response by microorganisms. Bacteriol. Rev. 39:121-143.
16. Shenker, B. J. 1987. Immunologic dysfunction in the pathogenesis of periodontal diseases. J. Clin. Periodontol. 14:489-498.
17. Shenker, B. J. 1988. Immunosuppression: an etiopathogenic mechanism, p. 178-186. In B. Guggenheim (ed.), Periodontology today. S. Karger, Basel.
18. Shenker, B. J., P. Berthold, P. Dougherty, and K. K. Porter. 1987. Immunosuppressive effects of Centipeda periodontii: selective cytotoxicity for lymphocytes and monocytes. Infect. Immun. 55:2332-2340.
19. Shenker, B. J., and J. M. DiRienzo. 1984. Suppression of human peripheral blood lymphocytes by Fusobacterium nucleatum. J. Immunol. 132:2357-2362.
20. Shenker, B. J., M. A. Listgarten, and N. S. Taichman. 1984. Suppression of human lymphocyte responses by oral spirochetes: a monocyte-dependent phenomenon. J. Immunol. 132:2039-2045.
21. Shenker, B. J., W. P. McArthur, and C. C. Tsai. 1982. Immune suppression induced by Actinobacillus actinomycetemcomitans. I. Effects on human peripheral blood lymphocyte responses to mitogens and antigens. J. Immunol. 128:148-154.
22. Shenker, B. J., and J. Slots. 1989. Immunomodulatory effects of Bacteroides products on in vitro human lymphocyte functions. Oral Microbiol. Immunol. 4:24-29.
23. Shenker, B. J., L. A. Vitale, and D. A. Welham. 1990. Immune suppression induced by Actinobacillus actinomycetemcomitans: effects on immunoglobulin production by human B cells. Infect. Immun. 58:3856-3862.
24. Shenker, B. J., W. F. Willoughby, M. R. Hollingdale, and T. N. Mann. 1980. In vivo responses to inhaled proteins. III. Inhibition of experimental immune complex pneumonitis after suppression of peripheral blood lymphocytes. J. Immunol. 124:1763-1772.
25. Smith, K. A. 1988. Interleukin-2: inception, impact and implications. Science 240:1169-1176.
26. Testi, R., J. H. Phillips, and L. L. Lanier. 1989. Leu 23 induction as an early marker of functional CD3/T cell antigen receptor triggering. Requirement for receptor cross-linking, prolonged elevation of intracellular Ca<sup>++</sup> and stimulation of protein kinase C. J. Immunol. 142:1854-1860.
27. Tomai, M. A., B. J. Elmquist, S. M. Warmka, and T. J. Fitzgerald. 1989. Macrophage mediated suppression of Con A induced IL-2 production in spleen cells from syphilitic rabbits. J. Immunol. 143:309-314.
28. Weir, D. M., and C. C. Blackwell. 1983. Interaction of bacteria with the immune system. J. Clin. Lab. Immunol. 10:1-12.